EXHIBIT 4

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Selective Infection and Cytolysis of Human Head and Neck Squamous Cell Carcinoma with Sparing of Normal Mucosa by a Cytotoxic Herpes Simplex Virus Type 1 (G207)

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ABSTRACT

This study evaluates inhibition of human squamous cell carcinomas (SCCs) by a replication-competent multimutated herpes simplex virus type 1 (G207). Infectivity and cytotoxicity of the G207 virus were evaluated *in vitro* in seven human SCC cell lines. *In vivo* effects of the G207 virus on human tumor xenografts in an athymic rat model were then investigated by injecting established tumors with 1×10^7 virus particles and monitoring tumor growth. In addition, oral cavity tumors in immunocompetent hamster were infected with the G207 virus by selective intraarterial perfusion and the tumor response was monitored. *In vitro* studies demonstrated infection rates, measured 24 hr after exposure, exceeding 40% at an MOI of 2 in five of seven human SCC cell lines. Cytotoxic effects, as measured by percent cell death on day 5, exceeded 90% in five of seven SCC cell lines. *In vivo* inhibition of tumor growth in an athymic rat model was seen ($p < 0.005$) and in two of the cell lines a complete clinical response was seen in 12 of 14 tumors. In the hamster model, selective intraarterial perfusion with G207 virus showed selective infection of the tumor cells, with sparing of the adjacent normal mucosa, which leading to significant suppression of tumor growth ($p < 0.005$). The G207 virus displayed efficient and selective cytotoxicity and tumor growth inhibition against human SCC and may prove useful as a therapeutic agent for head and neck SCC.

OVERVIEW SUMMARY

G207 is a cytotoxic herpes simplex virus type 1; it was designed for treatment of neurologic tumors and shares the natural neurotropism of all herpes viruses. The current study demonstrates that this virus has potent antitumor activity against noncentral nervous system human tumors *in vitro* and *in vivo*. It is effective when administered by either direct injection or regional perfusion. Regional intraarterial perfusion with G207 virus showed selective infection and killing of tumor cells, with sparing of the adjacent normal mucosa. G207 provides an attractive strategy for the treatment of solid tumors in that it is selective for rapidly dividing cells, can proliferate within a tumor, appears well tolerated in an animal model.

INTRODUCTION

SQUAMOUS CELL CARCINOMA (SCC) of the upper aerodigestive tract accounts for 42,800 new cancer cases in the United States per year (Vokes *et al.*, 1993). One-half of patients with SCC of the head and neck present with advanced stage disease (stage III or IV) and only approximately one-third of these patients survive 5 years with presently available therapies (Vokes *et al.*, 1993). In addition, significant functional morbidity, with respect to speech and deglutition, results from current treatment strategies of combined radical surgery, chemotherapy, and radiation therapy. The majority of patients with SCC of the head and neck fail locally or regionally, with only a small fraction failing distantly. This cohort of patients with advanced stage SCC of the head and neck awaits the development of novel ther-

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apies that improve local–regional control and ultimately survival while limiting functional morbidity.

Modified viruses have been studied in novel approaches for inhibiting tumor growth. Previous studies of genetically altered viruses for use in the treatment of SCC of the head and neck have employed replication-incompetent viral vectors as vehicles for transferring genes that may modulate tumor growth (Liu *et al.*, 1994; O'Malley *et al.*, 1995, 1996; Clayman *et al.*, 1996; Goebel *et al.*, 1996). Replication-competent engineered viruses that are inherently cytotoxic to tumor cells, however, offer an alternative approach. One such virus, the G207 virus, is a replication-competent herpes simplex type 1 (HSV-1) virus that has been modified to elicit specific tumor killing. This virus has multiple mutations including deletions at both loci of the γ34.5 gene, thus decreasing its neurovirulence; it retains the HSV thymidine kinase gene (HSV-*tk*) and has a *LacZ* insertion into the ICP6 gene (Chou *et al.*, 1990; Mineta *et al.*, 1995). The ICP6 gene encodes the large subunit of HSV ribonucleotide reductase, and loss of its expression decreases the ability of the G207 virus to proliferate in nondividing cells and increases specificity for tumor cell lysis. The various mutations in the G207 construct afford it several desirable characteristics, including the following: (1) decreased neuropathogenicity, (2) selectivity for rapidly dividing cells, (3) temperature sensitivity, (4) ganciclovir sensitivity, and (5) a marker gene (*lacZ*) (Mineta *et al.*, 1995). Previous studies have evaluated the efficacy of the G207 virus in treating neurologic tumors such as malignant gliomas, and have found its efficacy to be based on the natural neurotropism of the herpesvirus (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Miyatake *et al.*, 1997). The current study reports the effects of the replication-competent, cytotoxic HSV-1 virus G207 on nonneural tumors, specifically squamous cell carcinomas arising in the upper aerodigestive tract.

MATERIALS AND METHODS

Cell culture

Seven human SCC cell lines were utilized in this study. Five (HN 886, HN 921, MSK QLL2, MSK QLL1, and SCC 1483) were established by investigators from our institution (Memorial Sloan-Kettering Cancer Center, New York, NY) and two were purchased from the American Type Culture Collection (ATCC, Manassas, VA; SCC 15 and SCC 25). Cells were grown in minimal essential medium supplemented with 10% fetal calf serum under standard cell culture conditions.

G207 virus

The G207 virus was a gift from S. Rabkin (Georgetown University Medical Center) and was constructed as previously described with deletions of both copies of the γ34.5 gene and a *lacZ* gene insert into the ICP6 gene (Mineta *et al.*, 1995). The G207 virus was propagated in African green monkey kidney cells (Vero cells; ATCC), which were maintained in Dulbecco's modified Eagle's medium (DMEM). The Vero cells were infected with the G207 virus at a multiplicity of infection (MOI) of 0.02 at 34°C. The G207 virus was harvested after 2 days and subjected to freeze–thaw lysis to release virus from the cell fraction. Cell lysates were clarified by centrifugation (300 × *g* for 10 min at 4°C) and viral supernatants were aliquoted and stored at −80°C. Viral titers were determined on Vero cells by plaque assay.

In vitro: *X-Gal staining and cytotoxicity*

Each of the seven SCC cell lines was plated into 96-well microtiter plates at 3×10^4 cells per well in a volume of 100 μl. Twenty-four hours later the cells were then exposed to the G207 virus at MOIs of 0, 2, and 5 by adding 100 μl of the appropriate concentration of virus in medium to each well. On day 1, cells were stained with 5-bromo-4-chloro-3-indol yl-β-D-galactopyranoside (X-Gal) as previously described and the percentage of blue-staining cells calculated (Geller and Breakefield, 1988). In addition, cells were harvested from the wells by trypsinization and then counted, using a hemacytometer and trypan blue exclusion as the criterion for viability, on days 0, 1, 3, 5, and 7. Cell culture medium supernatant was also collected from each well and viral titers determined by assay of blue-forming units on Vero cells.

Cell cycle

Cell cycle analysis was performed on squamous cell carcinoma cells in culture. Cells were harvested by trypsinization, and cell suspensions were mixed in 80% ethanol and stored at −20°C until analysis. Prior to analysis cell suspensions were digested by DNase-free RNase for 20 min at 37°C and then stained in a propidium iodide solution (50 μg/ml). Cell cycle analysis was performed with a FACScan equipped with a FACStation running CellQuest software (Becton Dickinson, San Jose, CA). Debris was eliminated from analysis using a forward-angle light scatter threshold trigger. Cell doublets and other clumps were removed using analysis gates on either fluorescence pulse width or height versus pulse area (integral). Data for 10,000 to 20,000 single cells were collected per sample. Sub-G1 values were obtained by standard histogram analysis. Cell cycle analysis of DNA frequency histograms was performed with MultiCycle (Phoenix Flow Systems, San Diego, CA). Nuclear morphology of the cells stained with propidium iodide was also examined by fluorescence microscopy.

Inhibition of tumor growth in vivo

Human SCC xenograft in an athymic nude rat model. Human SSC xenografts in athymic nude rats were performed in accordance with and with the approval of the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Four- to 6-week-old athymic nude rats were maintained in a pathogen-free environment within the animal facility. The rats were anesthetized intraperitoneally with pentobarbital (50 mg/kg) and 2×10^6 tumor cells in 50 μl were injected subcutaneously into both left and right flanks of each rat. This yielded tumor nodules measuring approximately 5 mm in diameter by day 2 postintroduction. Between four and six separate flank tumors were established in each rat. When the tumors had reached approximately 5 mm in diameter, the experimental tumors were percutaneously injected with the 1×10^7 G207 virus particles diluted in culture medium (minimal essential medium supplemented with 10% fetal calf serum) to a volume of 50 μl, while the control tumors were injected percutaneously with 50 μl of heat-inactivated virus. The G207 virus was heat inactivated by

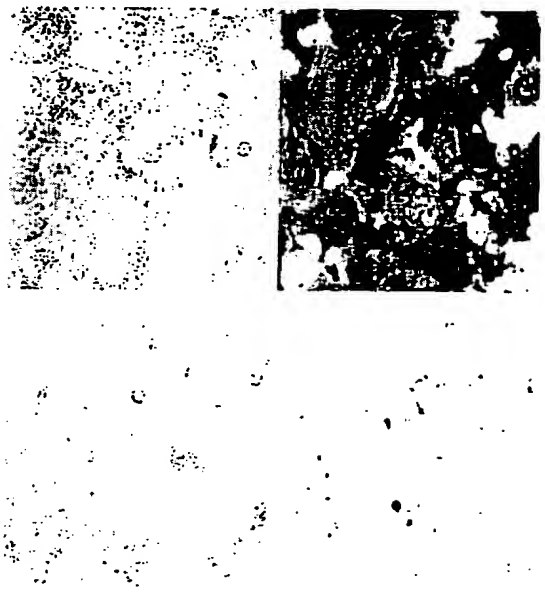
heating it to 80°C for 20 min. Heat-inactivated virus was used initially on the control tumors. Once it was determined, however, that there was no difference between the effects of heat-inactivated virus compared with culture medium alone (data not shown), carrier alone was used on the control tumors to conserve viral stocks.

Percutaneous injections were made with a single puncture but with multiple passes through the tumor nodule to maximize viral dispersion throughout the tumor. Tumor growth, i.e., the increase in the maximal diameter of the tumor in two axes, was measured and recorded three times a week. Volume was estimated by assuming the tumor volume to be that of a prolate spheroid with semiaxes a and b being one-half of the two maximal dimensions, and volume equal to (4/3) πab^2 (Selby, 1964). When animals were sacrificed tumors were stained with X-Gal as described above to determine the number of cells infected with the G207 virus, and also examined histologically using standard hematoxylin and eosin staining.

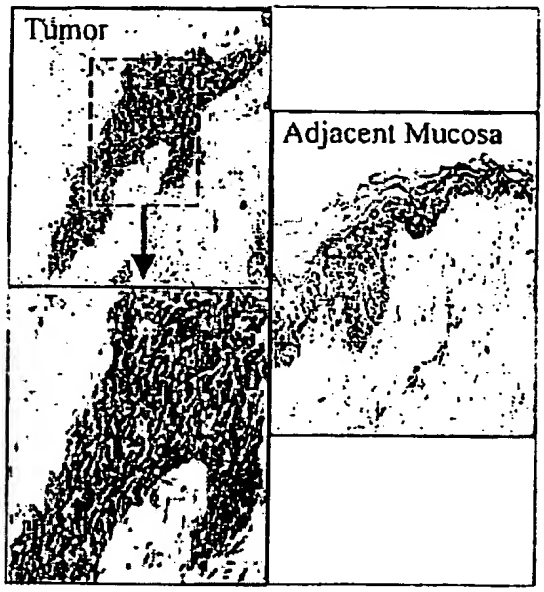
Experimental tumors generated by SCC cell line 1483, which was the most resistant to growth inhibition by the G207 virus, were injected with the G207 virus every 4 days for a total of three injections (control tumors were injected with culture medium, using the same schedule) to see if a multiple administration schedule improved results.

Selective intraarterial perfusion of oral cavity tumor. The carcinogen-induced hamster cheek pouch tumor was utilized as the model for the selective intraarterial perfusion studies. It should be noted that this is an immunocompetent animal model. Again, all procedures were performed in accordance with and with the approval of the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. To induce SCC tumors, the cheek pouches of Syrian golden hamsters were painted with a 0.5% solution of 7,12-dimethylbenz[*a*]anthracene dissolved in mineral oil three times a week for 16 weeks. The sizes of the tumors were monitored and when they had reached approximately 5–15 mm in greatest diameter they were entered into the study.

In preparation for the selective intraarterial perfusion of the cheek pouch tumors, the hamsters were anesthetized with pentobarbital (50 mg/kg) by intraperitoneal injection. The neck of the hamsters were prepped and a midline incision was made. The carotid vessels on the left were exposed and the common, internal, and external carotids were isolated. The remainder of the procedure was performed with the aid of an operating microscope. Microaneurysm clips were temporarily applied to obstruct arterial blood flow in the carotid system, the superior thyroid artery was ligated, and an arteriotomy was made in the proximal external carotid artery. A microcatheter system consisting of a blunt 30-gauge needle (Becton Dickinson) attached to the end of polyethylene tubing (internal diameter of 0.28 mm; Becton Dickinson) was inserted into the external carotid for approximately 5 mm. Previous studies with India ink perfusions



COLOR PLATE 1. Rate of *lacZ* expression as determined by X-Gal staining after infection with G207. *Top left:* X-Gal staining of SCC 15 control (not exposed to G207; original magnification, ×200). *Top right:* X-Gal staining of SCC 15, 1 day after exposure to the G207 virus at a multiplicity of infection (MOI) of 5 (original magnification, ×200). *Bottom left:* X-Gal staining of SCC 15 control (not exposed to G207; original magnification, ×200). *Bottom right:* X-Gal staining of SCC 15, 3 days after exposure to the G207 virus at an MOI of 5 (original magnification, ×200).



COLOR PLATE 2. Results of *in vivo* administration of G207 in an oral cavity tumor model. Chemically induced hamster cheek pouch tumors were selectively perfused via the external carotid artery with 1×10^7 G207 viral particles. Twenty-four hours later, tumors were harvested and stained with X-Gal to detect the marker LacZ. *Top and bottom left:* X-Gal staining of hamster cheek pouch tumor perfused with the G207 virus demonstrates a high rate of LacZ expression in the nest of neoplastic cells while sparing the nonneoplastic stroma (original magnifications, ×200 and ×400, respectively). *Right:* X-Gal staining of hamster cheek pouch mucosa adjacent to the tumor perfused with the G207 virus, showing absence of LacZ expression (original magnification, ×200).

have shown the external carotid artery to supply the hamster cheek pouch. A tie was placed around the cannulated external carotid artery to secure the catheter and prevent egress of infusate. The microaneurysm clip was removed from the distal external carotid artery and 250 μl of 25% mannitol (Abbott Laboratories, Abbott Park, IL) was perfused into the external carotid artery. The internal and external jugular veins on the right were then occluded to prevent vascular outflow and the 25% mannitol solution was allowed to dwell for 5 min. At this time 250 μl of carrier medium in the control group or 250 μl of the viral preparation containing 1×10^7 G207 viral particles in the experimental group was slowly perfused into the external carotid artery and allowed to dwell for 30 min. Once this dwell was complete, a microaneurysm clip was reapplied to the distal external carotid artery, the microcatheter was removed, and the arteriotomy was closed with 9-0 nylon suture (Ethicon, Sommerville, NJ). All microaneurysm clips were removed, the neck incision was then closed, and the animal allowed to recover.

Measurement of tumor growth, performed once a week, involved recording the maximal diameter of the tumor in three axes. Volume was again estimated to be that of an ellipsoid, with semiaxes a, b, and c being one-half the measured maximal diameters (Selby, 1964). The volume was thus calculated from the following formula: volume $= (4/3)\pi abc$ (Selby, 1964). When the tumors were excised, tumor specimens were stained with X-Gal as described above to determine the number of cells infected with the G207 virus, and were also examined histologically.

RESULTS

In vitro: *X-Gal staining*

To determine the rate at which the G207 virus infected the seven human squamous cell carcinomas, cells were infected at MOIs of 2 and 5 and X-Gal staining was performed 24 hr later to detect the marker gene (Color Plate 1). At an MOI of 2, more than 40% of the cells were infected with the virus in five of the seven cell lines (Table 1). The increase from an MOI of 2 to an MOI of 5 resulted in only a small increase in the percentage of cells infected with the G207 virus (Fig. 1).

In vitro: *Cytotoxicity*

The human SCC cell lines were exposed to the G207 virus at MOIs ranging from 0 to 10 and cell survival was monitored over 7 days. With increasing MOIs the cell death progressively increased, with nearly complete cell death by day 5 (Figs. 2 and 3). Even in cell lines with low infectivity, significant cell killing was noted.

In vitro: *G207 proliferation*

Supernatants were collected from the wells containing cells exposed to the G207 virus at an MOI of 5 (1.5×10^5 viral particles) to determine the rate by which the cell lines shed G207 virus into the supernatant during the period of treatment. The number of G207 viral particles detectable in the supernatant is seen to rise dramatically in only one cell line, SCC 15, while in the other three cell lines it remains at a relatively low level (Table 2). The production of significant amounts of G207 virus was found in the one cell line, SCC 15, that also demonstrates the greatest susceptibility to G207 cytotoxicity.

In vitro: *Cell cycle/DNA analysis*

The S-phase fraction obtained for the seven cell lines, under the subconfluent conditions in which they existed during the G207 cytotoxicity experiment, are listed in Table 1. It is again interesting to note that the cell lines with the lowest S-phase fraction tended to be those that were the most resistant to G207 infectivity and cytotoxicity, while those with the highest S-phase fraction were the most sensitive. When plotted in a log-linear fashion, the relationship between S-phase fraction and G207-mediated cytotoxicity correlated ($r^2 = 0.86$).

In vivo *inhibition of tumor growth*

Xenograft tumors were established in the subcutaneous tissue of the franks of athymic nude rats, using three cell lines (SCC 15, SCC 1483, and MSK QLL2). Once the tumors had

TABLE 1. CHARACTERISTICS OF THE VARIOUS CELL LINES TESTED[a]

Cell line	*S-phase fraction (c.v. G1)*	*Percent blue cells by X-Gal staining at 24 hr*	*Cell count 5 days after exposure to G207 at an MOI of 2*
HN 921	4.8% (c.v. G1 = 4.6)	46	55,200
SCC 25	18% (c.v. G1 = 4.9)	4.8	12,800
MSK QLL2	19% (c.v. G1 = 6.3)	70	5,070
SCC 15	23% (c.v. G1 = 5.1)	66	170
SCC 1483	29% (c.v. G1 = 3.9)	9.9	5,170
MSK QLL1	38% (c.v. G1 = 3.7)	93	0
HN 886	48% (c.v. G1 = 4.0)	93	1,200

Abbreviation: c.v., coefficient of variance.

[a]The S-phase fraction of the cells is listed in the second column. Cell lines are listed in order of increasing S-phase fraction. The percentage of cells staining blue on X-Gal staining 24 hr after exposure to G207 at an MOI of 2 is listed in the third column. The cell count of the remaining cells determined on day 5 after exposure to G207 at an MOI of 2 is listed in the fourth column. Initially, 3×10^4 cells were plated and controls showed exponential growth. All data represent the means of experiments performed in triplicate.

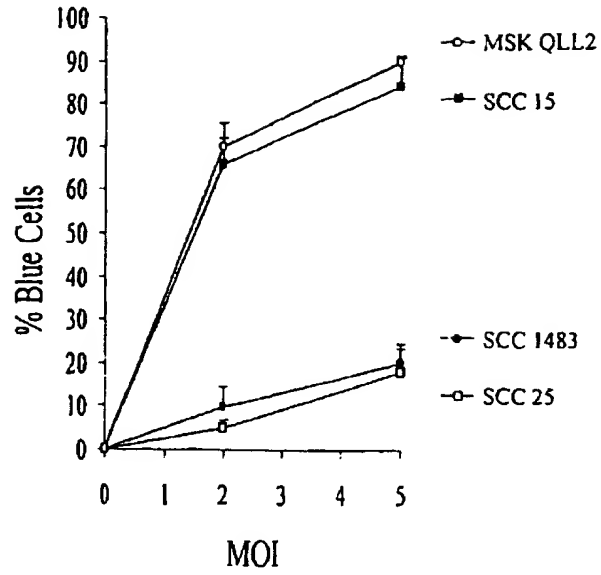


FIG. 1. Rate of infection of four representative human squamous cell carcinomas with G207, as measured by the expression of the marker gene *lacZ*. Human SCC cell lines were exposed to G207 at MOIs of 2 and 5 and then stained with X-Gal 24 hr later to determine the percentage of cells expressing the marker gene *lacZ*. Data plotted represent means ± standard errors of the mean.

reached approximately 5 mm in diameter, 1×10^7 G207 virus particles were injected percutaneously into the flank tumors. In tumors generated by two of the three cell lines, SCC 15 and MSK QLL2, a complete clinical response occurred: 12 of 14 tumor nodules could no longer be seen or palpated at 3 weeks. Most of these responses were durable, lasting up to 8 months; in addition, significant inhibition of growth was seen by 2 weeks after injection of the G207 virus ($p < 0.005$, unpaired t test; Fig. 4). Several athymic nude rats were sacrificed 3 weeks after injection of the G207 virus and showed persistent LacZ expression on X-Gal staining.

Concerning the cell line that was relatively resistant to *in vivo* tumor growth inhibition (SCC 1483), a schedule consisting of multiple repeated percutaneous injections of 1×10^7 viral particles every 4 days, to a total of three doses, was well tolerated and further improved the efficacy of the G207 virus in inhibiting tumor growth in the athymic nude rat model (Fig. 5).

Selective intraarterial perfusion of oral cavity tumor. Eight hamsters underwent selective intravascular perfusion of their left external carotid arteries, with either carrier medium (control; $n = 3$) or 1×10^7 G207 viral particles (experimental; $n = 5$). Two of the experimental animals were sacrificed at 24 hr and the bilateral cheek pouch tumors were harvested and stained with X-Gal to determine the rate of infection. Positive staining for LacZ was seen in a substantial proportion of the tumors cells in the cheek pouch tumors (Color Plate 2). Interestingly, the expression of the marker gene for G207 was preferentially found within the squamous carcinoma cells and spared normal mucosa adjacent to the tumor (Color Plate 2).

The selective intraarterial perfusion of the G207 virus into the hamster cheek pouch tumors showed an inhibition of tumor growth ($p < 0.005$; Fig. 6).

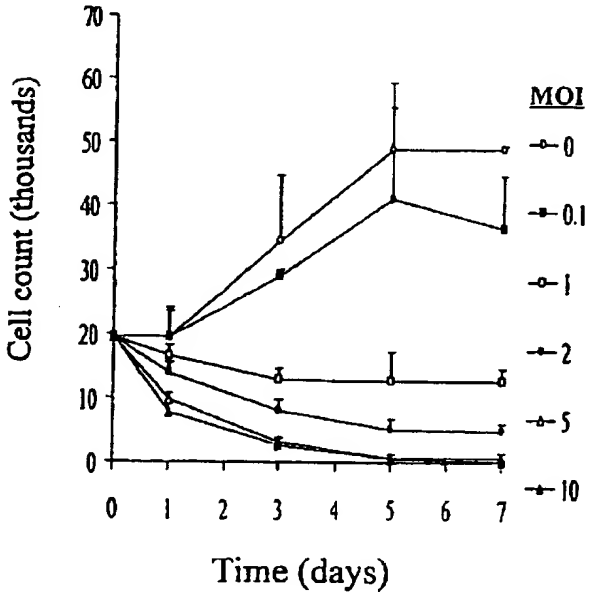


FIG. 2. *In vitro* inhibition of cell growth by G207 virus. Inhibition of cell growth of SCC 1483 at MOIs ranging from 0 (control) to 10, recorded on days 0, 1, 3, 5, and 7. A dose-dependent decrease in the number of cells over time is seen with an increasing MOI of G207. Data plotted represent means ± standard errors of the mean.

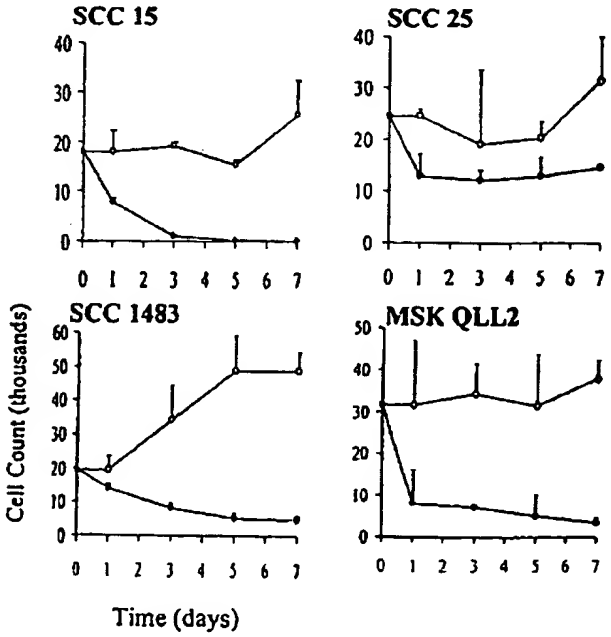


FIG. 3. Results of *in vitro* administration of G207 at an MOI of 2 to the four representative human SCC cell lines SCC 15, SCC 25, SCC 1483, and MSK QLL2. Open symbols represent the controls (not exposed to G207) and the closed symbols represent the cells exposed to G207 at an MOI of 2. Data plotted are the means ± standard errors of the mean.

TABLE 2. VIRAL TITER OF SUPERNATANTS COLLECTED FROM CELL LINES 1, 3, AND 7 DAYS AFTER EXPOSURE TO THE G207 VIRUS AT AN MOI OF 5[a]

Cell line	Number of G207 viral particles added on day 0	Viral titer		
		Day 3	Day 5	Day 7
SCC 15	150,000	9,770	41,300	704
SCC 25	150,000	3,290	2,930	2,630
SCC 1483	150,000	2,370	2,510	2,030
MSK QLL2	150,000	4,040	5,920	11,100

[a]Titers measured by assay of blue-forming units on Vero cells.

Histology

Serial sections of the hamster cheek pouch tumors were stained with X-Gal and adjacent sections were stained with hematoxylin and eosin. Cells that stained positive with X-Gal displayed nuclear staining that was disorganized, fragmented, and most consistent with a process dominated by necrosis.

DISCUSSION

The normal life cycle of the herpes simplex type 1 virus begins with an initial infection of a cutaneous or mucosal surface followed by a lytic replication cycle. The viral progeny from this initial lytic cycle can then infect neurons that innervate the epithelial site of viral entry. These characteristics of the HSV-1 virus, namely, its cytolytic activity and its neurotropism, have stimulated research into the use of genetically engineered mutants of HSV-1 for the treatment of neural tumors. Initially, single-gene mutants of the HSV-1 virus were engineered with attenuated neurovirulence (Chou *et al.*, 1990). More recently, HSV-1 vectors such as the G207 virus have been constructed with mutations of multiple genes to maximize both the cytotoxic effects as well as the clinical safety of these vectors (Mineta *et al.*, 1995). The G207 virus has multiple mutations, including deletions at both loci of the γ34.5 gene and a β-galactosidase (LacZ) insertion into the ICP6 gene. The deletions of the γ34.5 gene decrease its neurovirulence while the insertion into the ICP6 gene, which encodes the large subunit of HSV ribonucleotide reductase, decreases its ability to proliferate in

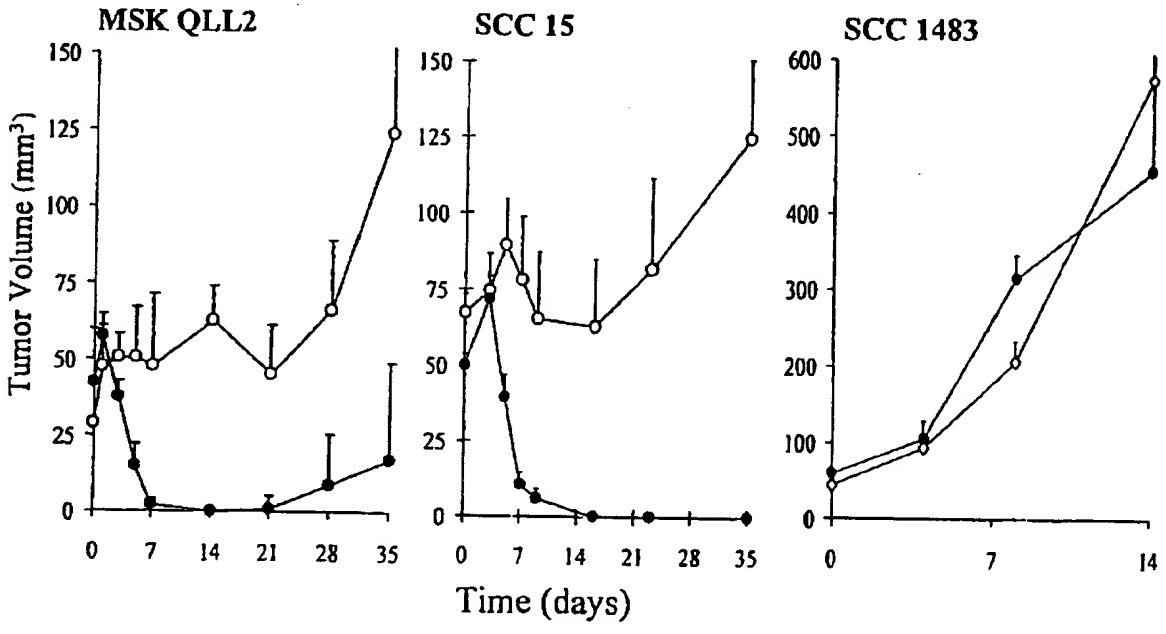


FIG. 4. Summary of *in vivo* inhibition of flank tumor xenografts of the three tumorigenic cell lines MSK QLL2, SCC 15, and SCC 1483 in an athymic nude rat model. A single percutaneous injection of 1×10^7 G207 viral particles was administered on day 0. A minimum of six tumors was tested in each experimental group. Open symbols represent the controls (injected with carrier medium) and closed symbols represent the tumors injected with 1×10^7 G207 viral particles. Data plotted are the means $\pm$ standard errors of the mean.

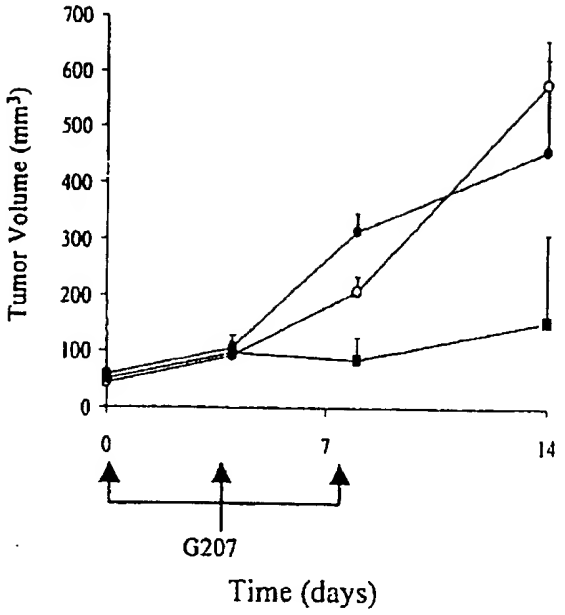


FIG. 5. Results of *in vivo* inhibition of the SCC 1483 flank tumor xenografts in an athymic nude rat model after multiple injections of G207. A total of three percutaneous injections of 1×10^7 G207 viral particles was administered on days 0, 4, and 8. Open circles represent the controls (injected with carrier medium), the closed circles represent the tumors injected a single time with 1×10^7 G207 viral particles and the closed squares represent the tumors injected multiple times with 1×10^7 G207 viral particles. Data plotted are the means ± standard errors of the mean.

cells not undergoing rapid cell division. The clinical safety of the G207 virus is further augmented by its exquisite sensitivity to the antiherpetic drug ganciclovir, which can be used as a rescue. The multiple mutations of the G207 virus also minimize the chance of reversion to the wild-type HSV-1. The neurotropic characteristics of HSV-1 have led to many investigations of mutants of this virus in the treatment of neural tumors (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Miyatake *et al.*, 1997). These studies have demonstrated the ability of the HSV-1 mutants to inhibit malignant brain tumor growth *in vivo*. Preliminary studies using a related, replication-competent herpesvirus (HSV 1716) that has a deletion in the γ34.5 gene have demonstrated tumor growth inhibition in human malignant mesotheliomas (Kucharczuk *et al.*, 1997). Few studies, however, have evaluated the ability of the multimutated G207 virus to inhibit the growth of nonneural solid tumors.

The ability of the HSV-1 virus to infect epithelial sites and enter a lytic cycle suggests that it may be a useful agent in the treatment of tumors of epithelial origin, such as SCC of the upper aerodigestive tract. In the current studies the G207 virus displays efficient *in vitro* and *in vivo* infection and lysis of all human SCCs evaluated. The *in vivo* effects of the G207 virus correlate with the *in vitro* results, in that the cell line that was most sensitive in cell culture (SCC 15) was also the most sensitive in the athymic nude rat model. The data presented in this article indicate that the G207 virus efficiently infects and lyses human SCC tumor cells and may provide a novel therapy in the treatment of the patients with these tumors. In addition, this study demonstrated that *in vitro* sensitivity assays as well as proliferative indices of tumor cells may also be used to predict clinical efficacy.

Introducing the virus by selective intravascular perfusion is appealing in that it allows a diffuse distribution of the virus within the tumor. The same hypervascularity that the tumor creates to allow continued growth can also be used to increase virus delivery. G207 is a large virus and there was concern as to whether such a large particle may cross the endothelium and enter tumor cells. The hamster cheek pouch perfusion experiment indicates that G207 virus is able to cross the endothelium and infect tumor cells. Interestingly, in the hamster cheek pouch model, in which the G207 virus was introduced by intraarterial perfusion, the G207 virus appeared to infect selectively the neoplastic cells while sparing the normal adjacent mucosa, despite the high rate of proliferation within the basal layer of the epithelium.

The prospect of the use of a replication-competent virus as a possible anticancer agent does raise concern with regard to its safety in future clinical trials. To improve its safety relative to earlier generation HSV-1 mutants, the G207 virus has been designed with several safeguards. First, the G207 virus is multimutated so that reversion to wild-type HSV-1 is minimized. In addition, the G207 virus is exquisitely sensitive to ganciclovir, thus allowing a potential means of eradicating the G207 virus if it were to become a source of systemic morbidity. Finally, the various mutations decrease its neurotoxicity and render it unable to proliferate efficiently within nondividing cells.

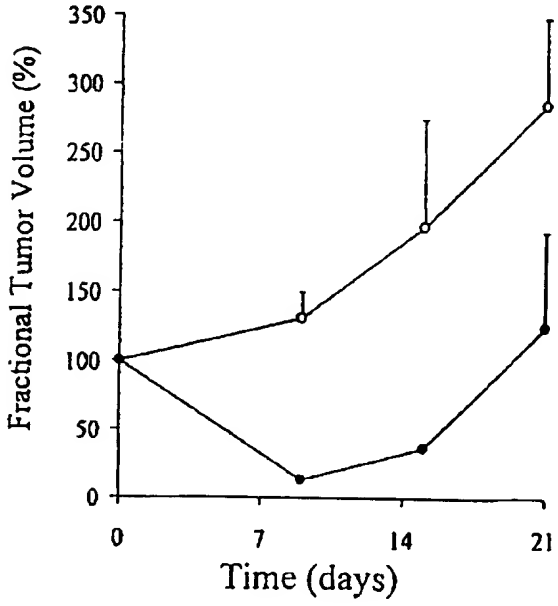


FIG. 6. *In vivo* inhibition of hamster cheek pouch tumor growth after selective intraarterial perfusion of the external carotid artery with 1×10^7 G207 viral particles. Open circles represent the controls (perfused with carrier medium) and the closed circles represent the tumors, which were selectively perfused with 1×10^7 G207 viral particles. Data plotted are the means ± standard errors of the mean.

In the two *in vivo* models utilized in this study, neither short- nor long-term morbidity or mortality was observed as a result of the administration of G207 either by subcutaneous injection or by intravascular perfusion. Previous investigations have also demonstrated the G207 virus to be avirulent after intracerebral inoculation in HSV-sensitive nonhuman primates (Mineta *et al.*, 1995).

The majority of patients with SCC of the upper aerodigestive tract continue to present with advanced stage disease. Despite aggressive treatment paradigms, including surgery, radiation therapy, and chemotherapy, fewer than half of these patients survive 5 years. Clearly new treatment approaches for these patients are needed. This study provides evidence that the multimutated, cytotoxic, replication-competent HSV virus G207 has *in vitro* and *in vivo* antitumor activity against non-central nervous system tumors. It provides an attractive strategy for the treatment of solid tumors in that it can proliferate within a tumor and appears well tolerated in an animal model when introduced locally and intravascularly. The G207 virus may therefore be a useful agent in the treatment of solid human malignancies in general, and these data would encourage human trials using oncolytic viruses in the treatment of human head and neck cancer.

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